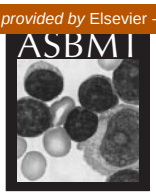
Biology of Blood and Marrow Transplantation 8:360-367 (2002)



Effect of c-mpl Ligands after Total Body Irradiation (TBI) with and without Allogeneic Hematopoietic Stem Cell Transplantation: Low-Dose TBI Does Not Prevent Sensitization

Richard A. Nash,[1] *Alessandra Takatu,*[1] *Ziding Feng,*[1] *Sherrill Slichter,*[2] *Kraig Abrams,*[2] *German Espino,*[1] *M. John Gass,*[1] *George E. Georges,*[1] *Peter A. McSweeney,*[1] *Howard M. Shulman,*[1] *Rainer Storb*[1]

[1]Fred Hutchinson Cancer Research Center and the [2]Puget Sound Blood Center, Seattle, Washington

Correspondence and reprint requests: Richard A. Nash, MD, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave N, D1-100, PO Box 19024, Seattle, WA 98109-1024 (e-mail: rnash@fhcrc.org).

Received October 18, 2001; accepted April 19, 2002

ABSTRACT

This study investigates the potential role of the recombinant c-mpl ligands (recombinant human thrombopoietin [rhTPO] and pegylated recombinant human megakaryocyte growth and development factor [PEG-rhMGDF]) on the recovery of platelet counts after TBI with and without allogeneic hematopoietic stem cell transplantation (HSCT) in an established canine model. Initially, 3 cohorts, each with 2 nonirradiated dogs, received increasing doses of rhTPO (5 μg/kg per day; 10 μg/kg per day; 20 μg/kg per day) for 7 days to determine the optimal dose. The dose of 10 μg/kg per day of rhTPO was selected for subsequent studies. Ten dogs then received either rhTPO or placebo for 28 days after 200 cGy TBI without HSCT. The rhTPO group had fewer days with platelet counts <20,000/μL (9.8 days versus 17.8 days, $P < .05$) and significantly increased granulocyte counts (n = 5) compared to the controls (n = 5). RhTPO-specific antibodies developed in 2 dogs, which caused a significant but transient decrease of the platelet counts. Retreatment of these sensitized dogs with rhTPO resulted in profound transient decreases in platelet counts. In the next study, 20 dogs received either PEG-rhMGDF or placebo for 21 days after 920 cGy TBI and allogeneic HSCT. The median time to platelet recovery (>20,000/μL) for the PEG-rhMGDF group (n = 10) was 14.0 days compared to 15.5 days for the control group (n = 10; log rank, $P = .35$). There were no significant differences in the total time to platelet counts <20,000/μL or in the time to recover neutrophil counts >500/μL. The effects of rhTPO on recovery of platelet and granulocyte counts after sublethal TBI were modest, and no effects of PEG-rhMGDF were observed on hematopoietic recovery after high-dose TBI and allogeneic HSCT. The significant effect that rhTPO-specific antibodies had on the platelet counts may limit the clinical role of recombinant c-mpl ligands unless sensitization can be prevented.

KEY WORDS

Thrombopoietin • rhTPO • PEG-rhMGDF • Dogs • Platelet recovery • Total body irradiation • Sensitization • Antibodies • c-mpl ligand

INTRODUCTION

Severe persistent thrombocytopenia after myeloablative conditioning regimens and hematopoietic stem cell transplantation (HSCT) increases the risk of life-threatening hemorrhage and requires close monitoring and support with blood products [1-6]. Continuous support with platelet transfusions is associated with morbidity related to transfusion reactions, platelet allo-immunization, and transfusion-related viral infections. There is also a significant financial burden associated with platelet transfusion support [2]. New treatments that could decrease the period of thrombocytopenia could have significant clinical benefits [7-10].

The ligand for c-mpl is a critical cytokine that regulates platelet numbers in the peripheral circulation [11]. In c-mpl ligand and c-mpl knock-out mice, there is a marked reduction

P.A.M. is currently with University of Colorado Health Sciences Center, Denver, CO.

but not an absence of marrow megakaryocytes and platelets [12]. In humans, mutations of c-mpl associated with a loss of function cause congenital amegakaryocytic thrombocytopenia [13]. The ligand for c-mpl is relatively lineage specific, works both alone and synergistically with other cytokines to support early hematopoietic progenitor production and megakaryocyte colony formation, and acts at a late stage of development to increase megakaryocyte size, polyploidization, and expression of differentiation markers [14-18]. Two forms of recombinant human c-mpl ligand were developed for clinical evaluation: the full-length molecule known as recombinant human thrombopoietin (rhTPO), and a truncated version, bound to polyethylene glycol and known as pegylated recombinant human megakaryocyte growth and development factor (PEG-rhMGDF). Both recombinant forms have shown potent platelet stimulatory activity and excellent clinical tolerance in different species [7,8,10,19-21]. In vivo, treatment with c-mpl ligands (rhTPO and PEG-rhMGDF) has increased platelet counts and expanded the numbers of megakaryocytes and their progenitors in the marrow and spleen [14].

In this study, the effect of recombinant forms of c-mpl ligand on recovery of platelet counts after a sublethal dose of a cytotoxic agent (total body irradiation [TBI] 200 cGy) without HSCT and after a high-dose conditioning regimen (TBI 920 cGy) with HSCT was investigated in an established canine transplantation model [22]. RhTPO-specific antibodies developed in some dogs, which caused a significant decrease of platelet counts.

MATERIALS AND METHODS

Experimental Animals

Forty-one random-bred dogs, 6 to 18 months of age and weighing 6 to 15 kg, were entered into the study. The dogs were either raised at the Fred Hutchinson Cancer Research Center (FHCRC) or obtained from breeders licensed by the U.S. Department of Agriculture. Dogs were quarantined on arrival, screened for evidence of disease, and observed for a minimum of 2 months prior to entering into protocol. They were dewormed and vaccinated for rabies, distemper, leptospirosis, papillomavirus, hepatitis, and parvovirus. They were housed in an American Association for Accreditation of Laboratory Animal Care accredited facility in standard indoor runs and were provided commercial dog chow and chlorinated tap water ad libitum. Animal holding areas were maintained at 21°C ± 1°C with 50% ± 10% relative humidity using at least 15 air changes per hour of 100% conditioned fresh air. The dogs were on a daily 12-hour light/dark full-spectrum lighting cycle (LD12:12) with no twilight. The Institutional Animal Care and Use Committee of FHCRC approved the protocols for this study.

Thrombopoietin (rhTPO and PEG-rhMGDF)

RhTPO was provided by Zymogenetics (Seattle, WA). Increasing doses of rhTPO were administered by subcutaneous injection twice a day for 7 days in 6 nonirradiated dogs (5 μg/kg per day; 10 μg/kg per day; 15 μg/kg per day). In 5 dogs, rhTPO (10 μg/kg per day) was administered for 28 days starting within 2 hours of a sublethal dose of TBI (200 cGy). Five dogs received only vehicle as part of the control group.

Two dogs that developed rhTPO-specific antibodies were re-treated with 5 or 10 μg/kg per day rhTPO beginning after platelet counts had recovered after TBI.

Next, PEG-rhMGDF was provided by Amgen (Thousand Oaks, CA) for studies of platelet recovery after allogeneic HSCT. Ten dogs were treated with PEG-rhMGDF by subcutaneous injection (10 μg/kg per day, divided) twice a day for 21 days, starting within 2 hours of high-dose TBI (920 cGy). This biologically active dose of PEG-rhMGDF was based on previous studies in dogs [23]. Ten dogs received only the vehicle as part of the control group.

TBI, Marrow Transplantation, and Postirradiation Care

Ten dogs were given 200 cGy TBI and 20 dogs were given 920 cGy TBI in a single fraction at 7 cGy/min from 2 opposing ^{60}Co sources [22]. The day of TBI was designated as day 0. For those dogs that received HSCT after 920 cGy TBI, a total nucleated cell (TNC) dose of 4.0×10^8 TNC/kg of marrow from a dog leukocyte antigen [DLA]–identical littermate was targeted for infusion. After TBI, parenteral fluids, electrolytes, platelet transfusions, and broad-spectrum antibiotics were administered as described. After HSCT, the intravenous formulation of cyclosporine (20 mg/kg per day) was administered in 2 divided doses from days 0 to 6. The oral formulation of cyclosporine (30 mg/kg per day) was administered in 2 divided doses from days 7 to 42 [22]. All blood products used for transfusions were irradiated in vitro (1500 cGy) to inactivate immunologically competent cells. Autopsies with histologic examinations of the organs were performed on all dogs that died during the study period.

DLA Typing of Canine Littermates

Ten matched littermate donor/recipient pairs were chosen for studies of allogeneic HSCT on the basis of identity for highly polymorphic major histocompatibility complex (MHC) class I and class II microsatellite markers [24,25]. Specific DLA DRB1 allelic identity was determined by direct sequencing [26].

Hematology

Hematology examinations consisting of complete blood counts and white blood cell count (WBC) differentials were performed at least twice before administration of TPO/PEG-rhMGDF and for a minimum of 5 days per week subsequently. The samples were collected into a tube containing EDTA. Automated hematological analyses of blood samples were done on a Sysmex E 2500 (Baxter, Chicago, IL). Blood smears were stained with Wright-Giemsa for WBC differentials.

Marrow Biopsy, Aspiration, and Liver Biopsy

For bone marrow evaluations, dogs were anesthetized by intravenous injection of fentanyl (0.4 mg/9 kg; Innovar-Vet, Pitman-Moore, Mundelein, IL), ketamine hydrochloride (42 mg/9 kg) and acepromazine (1.7 mg/9 kg; Aveco, Fort Dodge, IA). Marrow aspiration and biopsy specimens were obtained before and 28 days after administration of TPO/PEG-rhMGDF. Liver biopsies were performed under anesthesia and ultrasound guidance if platelet count was above 60,000/μL.

Platelet Survival

All assessments of platelet survival were done with autologous platelets labeled with chromium-51 [27]. Venous blood was obtained at 1 hour after infusion of labeled platelets and then daily for 4 days to determine the rates of disappearance of platelet radioactivity. Platelet survival was measured in days. An assessment of platelet survival was done prior to administration of rhTPO to establish a baseline value for each animal to compare with measurements obtained weekly for 3 weeks after the start of TPO.

Serum Chemistry and Plasma Fibrinogen Levels

Serum samples from nonirradiated dogs that received increasing doses of rhTPO were collected on day 0 before the administration of TPO/PEG-rhMGDF and thereafter on days 7, 14, and 21. Serum concentrations of sodium, potassium, chloride, total CO_2, creatinine, glucose, calcium, phosphorus, urea nitrogen, uric acid, cholesterol, triglyceride, albumin, globulin, total bilirubin, direct bilirubin, lactic dehydrogenase (LDH), glutamic-pyruvic transaminase (SGPT), γ-glutamyl transaminase (SGOT), γ- glutamyl transpeptidase (GGT), and alkaline phosphatase were measured. Fibrinogen levels were measured in plasma using the thrombin clotting time technique. The change in light scatter due to fibrin clot formation after the addition of Bovine Thrombin 500 (Pacific Hemostasis, Houston, TX) was measured in a MLA Electra 800 (Baxter).

Study of Immunized Dogs with rhTPO

Dogs that developed rhTPO-specific antibodies after the initial treatment regimen were re-treated with rhTPO to boost the immune response. Complete blood counts were followed serially to assess the effect of retreatment with rhTPO, and plasma was collected after the development of thrombocytopenia. Plasmapheresis was performed for 5 days, and a minimum 32 mL/kg was collected from an rhTPO-sensitized, thrombocytopenic dog (D978) and 2 untreated dogs. The plasma was stored in a –70°C freezer. Red blood cells were reconstituted with saline 9% and infused into the dog from which they had been drawn. Plasma from the untreated (control) and from the rhTPO-sensitized dogs was infused into untreated, normal recipient dogs at 16 to 20 mL/kg per dose, intravenously, on days 0 and 1. Serial complete blood counts were performed. Serology studies were performed to confirm the presence of rhTPO-specific antibody.

Methodology for Assessing rhTPO-Specific or PEG-rhMGDF–Specific Antibodies

An enzyme-linked immunosorbent assay (ELISA) was established to monitor for the presence of rhTPO-specific antibodies in dog plasma. Ninety-six–well microtiter plates were coated with rhTPO at 250 ng/mL diluted in coating buffer (0.1 mol/L Na_2HCO_3, pH 9.6). The plates were incubated overnight at 4°C, washed with ELISA C buffer (phosphate-buffered saline [PBS], 0.05% Tween-20), then blocked with ELISA B buffer (PBS, 0.1% bovine serum albumin [BSA], 0.05% Tween-20). Test samples of dog plasma were diluted in ELISA B buffer, added to the wells, and incubated at 37°C for 2 hours. The test samples were removed, the wells were washed with ELISA C, and goat antidog immunoglobulin G conjugated to horseradish peroxidase (Cappell, Melvern, PA) diluted 1:2000 in ELISA B was added to the wells for 1 hour at 37°C. The wells were washed with ELISA C, then incubated with OPD substrate solution (12.5 mL 0.1 mol/L Na citrate, pH 5.0, 5 mg o-phenylenediamine, and 5 μL 30% H_2O_2). The reaction was stopped by the addition of 1N H_2SO_4, and the plates were read at absorbance 490 nm in a Dynatech ELISA plate reader (Molecular Devices, Sunnyvale, CA).

Dogs that received PEG-rhMGDF had their samples tested in a radioimmunoassay (RIA) and neutralizing antibody bioassay (modified from [23]). Data from the RIA were expressed as the ratio of the postinjection sample over the preinjection (baseline) sample. A reactive sample was one with a ratio >2. All of the samples submitted had post/pre ratios of less than 2 and were considered nonreactive. The results of the neutralizing antibody bioassay were expressed as titers. A titer of <1:50 was considered negative for neutralizing antibodies.

Statistical Analysis

To test whether the TPO group had fewer days in which platelet counts were below 20,000/μL, we used a randomization test [28]. For 2 dogs in each pair, the labels of TPO and control would be discarded when TPO had no effect on engraftment. Therefore, 2 possible values of the difference between 2 platelet counts could be obtained by switching the labels within the pair. There was a total of 32 possible combinations of such switches for 5 pairs. We computed the mean of the differences for each of the 32 combinations and ranked them. The 1-sided P value was the position of the observed difference from the data divided by 32. To examine the time trend for each group of dogs, a bootstrap method was used in which all of the possible 3125 combinations of the resampling pairs were computed [29]. The 90% confidence band for each group was computed by taking the lower 5% and upper 5% (rounded to integer) of the ranked mean platelet measures for each group. This result formed the 90% confidence band. To examine whether 2 groups had different time trends, we first computed the difference within each pair for each day and then bootstrapped on the pair differences to form the 90% confidence band. A $\log(x + 1)$ transformation for platelets and granulocytes was used so that the confidence band for the difference in the 2 groups at the original scale (base 10) was the logarithm of the ratio of the 2 groups at the original scale (TPO/control). For any day, the TPO group was judged to have significantly higher counts than the control group (1-sided test, $P < .05$) if the lower limit of the 90% confidence band was above 1 on that day.

A Wilcoxon's paired signed rank test was used to compare the effects of PEG-rhMGDF on platelet recovery after transplantation with the control group [30].

RESULTS

Effect of Increased Doses of rhTPO

Six normal dogs were treated with twice-daily injections of increasing doses of rhTPO (5, 10, and 20 μg/kg per day) subcutaneously for 7 days. All 6 dogs showed an increase in platelet counts (Figure 1). The 5 μg/kg per day dose of rhTPO was not as effective for increasing the platelet count

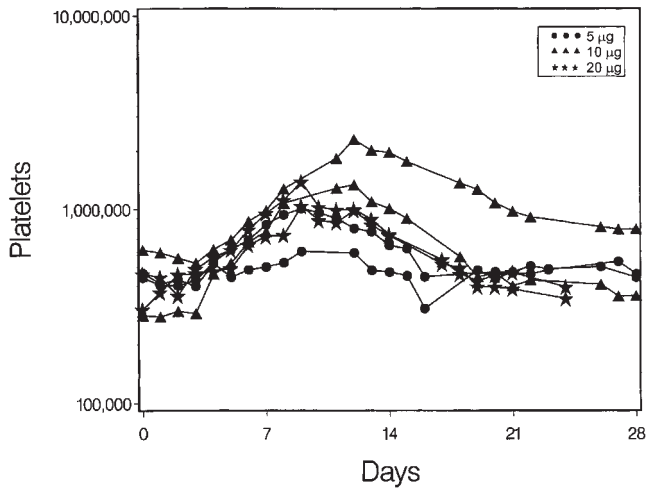


Figure 1. Effect on platelet counts after treatment of 6 normal dogs with twice-daily injections of increasing doses of rhTPO (5, 10, and 20 µg/kg per day) subcutaneously for 7 days.

as were the 2 higher doses. There was no apparent difference in peak platelet counts at the doses of 10 and 20 µg/kg per day. No significant changes in hematocrit, neutrophil, or monocyte counts were observed after treatment compared to baseline. An increase in serum LDH was observed (2- to 3-fold compared to baseline) with no other significant change in serum chemistries, including albumin and cholesterol. Platelet survival was unchanged compared to baseline during rhTPO treatment.

Effect of rhTPO on Platelet Recovery after 200 cGy TBI

Ten dogs were given 200 cGy TBI. Dogs were irradiated in matched pairs, of which one received 10 µg/kg per day rhTPO for 28 days and the other received vehicle only (TPO group, n = 5; control group, n = 5). The rhTPO group had fewer days with platelet counts <20,000/µL (9.8 days versus 17.8 days, P = .031) and fewer days with platelet counts <10,000/µL (4.4 days versus 14.4 days, P = .023) (Figure 2). Platelet counts in the rhTPO group were significantly increased compared to those of the controls between days 11 and 25 after TBI. Granulocyte counts in the rhTPO group were also significantly greater than those of the control group between days 13 and 25 after TBI (Figure 3). No significant difference in median hematocrit from day 0 to day 28 was observed between the 2 groups (P = .095). One control dog died on day 27 after TBI from infectious complications while pancytopenic.

Effect of PEG-rhMGDF on Platelet Recovery after 920 cGy TBI and Allogeneic Marrow Transplantation

Ten pairs of dogs received transplants of DLA-identical marrow from littermates after 920 cGy of TBI. The median cell doses infused were 3.89×10^8 TNC/kg (range, 2.09-4.15×10^8 TNC/kg) and 4.00×10^8 TNC/kg (range, 2.06-4.39×10^8 TNC/kg) for the PEG-rhMGDF and control groups, respectively. PEG-rhMGDF was given in 2 divided doses of 10 µg/kg per day, subcutaneously, for 21 days. The median time to a sustained recovery of platelet counts ≥20,000/µL for the PEG-rhMGDF group was 14.0 days, compared to 15.5 days for the placebo group (log rank, P = .35). There was no difference in the number of days that platelet counts were <20,000/µL (Figure 4). There was no significant difference between the 2 groups in the time to recover neutrophil counts >500/µL or in the hematocrit. No complications associated with PEG-rhMGDF were noted. There was no difference between the 2 groups in the occurrence of graft-versus-host disease (GVHD), and no GVHD occurrence was severe.

One dog in the PEG-rhMGDF group died from infectious complications while pancytopenic on day 19. Two dogs died in the control group (at days 36 and 45), and 3 dogs died in the PEG-rhMGDF group (at days 33, 40, and 41) with liver necrosis after platelet recovery. The necrosis involved the centrilobular and midzone areas of the liver. There was significant lysis of hepatocytes with minimal hemorrhage and residual stromal cells. No thrombosis was observed, and there was no evidence of veno-occlusive disease of the liver. It is interesting to note that of the 4 dogs that had routine liver biopsies performed at 6 to 10 days before death, 3 had no evidence of liver necrosis identified. The etiology for the liver necrosis was uncertain but may have been related to a viral hepatitis.

TPO Sensitization

There was no evidence of sensitization to rhTPO in any of the nonirradiated dogs that received increasing doses. All of these dogs were followed for a minimum of 4 weeks after completing rhTPO. Two of 5 dogs that received rhTPO after TBI (200 cGy) were sensitized and had either a markedly delayed recovery of platelet counts or a late decrease in platelet counts after an initial recovery. ELISA confirmed the presence of rhTPO-specific antibodies in these 2 dogs. No PEG-rhMGDF–specific antibodies were identified at day 28 after allogeneic HSCT.

The 2 rhTPO-sensitized dogs were re-treated with rhTPO after their platelet counts had recovered to normal

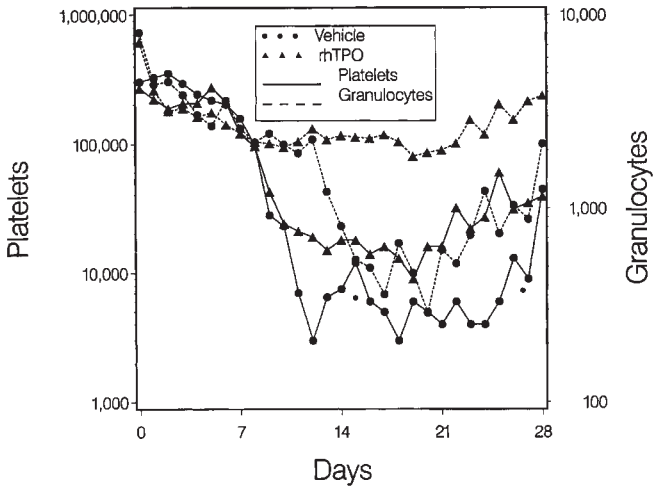


Figure 2. Effect of rhTPO on platelet and granulocyte recovery after TBI (200 cGy). Ten dogs were irradiated in matched pairs, of which one received 10 µg/kg per day rhTPO for 28 days and the other received vehicle only (TPO group, n = 5; control group, n = 5). Serial platelet and granulocyte counts are presented as median values.

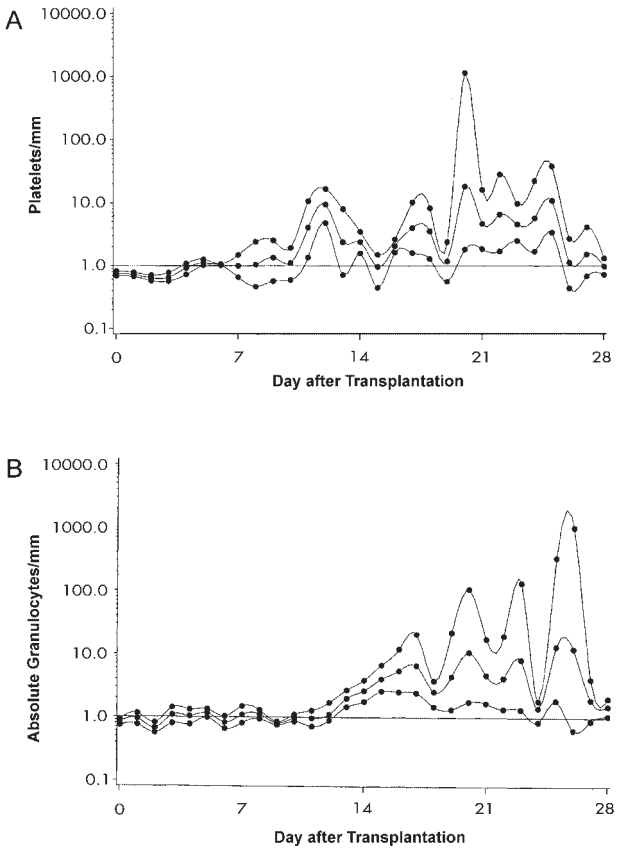


Figure 3. Ninety percent confidence band for the difference between treatment and control groups. For examination of the time trend of platelet and granulocyte recovery after TBI (200 cGy) for each group of dogs, a bootstrap method was used. The 90% confidence band (upper and lower lines) for each group was computed by taking lower 5% and upper 5% of the ranked mean platelet measures for each group. For any day, the TPO group was judged to have significantly higher counts than the control group (1-sided test, $P < .05$) if the low limit of the 90% confidence band was above 1 on that day. Platelet (A) and granulocyte (B) counts in the rhTPO group were significantly increased compared to those of the controls, predominantly between days 11 and 25 and between days 13 and 23 after TBI, respectively.

levels. The first retreatment of the 2 rhTPO-sensitized dogs (D978, E080) with rhTPO 10 μg/kg per day, 7 days apart for a total of 2 days, resulted in the recurrence of thrombocytopenia with platelet counts as low as 16,000/μL and 61,000/μL, respectively (Figure 5). Platelet counts persisted <100,000/μL for 13 and 3 weeks after the retreatment, respectively. During the period of thrombocytopenia induced by the administration of rhTPO, platelet survival in the peripheral circulation was normal. Results of serial multiple marrow biopsies showed that the megakaryocyte content was normal or reduced to between 10% to 30% of normal in dogs E080 and D978. Plasma from dog D978 and from untreated dogs was infused into normal dogs to evaluate the effect on platelet counts. There was a gradual modest reduction in platelet counts in the 3 dogs infused with plasma from D978. The 3 dogs had baseline platelet counts of 323,000/μL, 167,000/μL, and 337,000/μL and nadirs of 125,000/μL at day 17, 50,000/μL at day 29, and 84,000/μL at day 25, respectively. No significant changes in platelet counts were associated with the infusion of normal plasma. There was no change in platelet survival after plasma infusion.

DISCUSSION

The recombinant ligands for c-mpl (rhTPO and PEG-rhMGDF) have been used in preclinical models of myelosuppression and in clinical studies of myelosuppression to evaluate their effect on hematopoietic reconstitution. Although the study was initiated with rhTPO in normal dogs and in the low-dose TBI model, studies of platelet recovery after allogeneic HSCT were done with PEG-rhMGDF. There have been no direct comparisons between rhTPO and PEG-rhMGDF, but both molecules have the same c-mpl binding site, which is critical for the biological activity of the ligand. PEG-rhMGDF had previously been studied in dogs and was known to be biologically active [23]. The dose of PEG-rhMGDF (10 μg/kg per day for 21 days) in the study was at least comparable and probably several-fold more biologically active than the dose of rhTPO shown to be active after low-dose TBI in the earlier phase of this study [7,31]. Comparable doses and schedules of these 2 agents have been used in these previously reported preclinical studies. Administration of recombinant ligands for c-mpl to normal animals following myelosuppressive therapy with TBI or chemotherapy has been shown to decrease the severity and shorten the duration of thrombocytopenia as well as, in some cases, neutropenia [7,8,10,14,19-21,31-34]. The improved recovery of both platelet and granulocyte counts with rhTPO treatment after TBI (200 cGy) in dogs is consistent with these previous studies of rhTPO after myelosuppressive therapy in other species. Clinical studies of recombinant c-mpl ligand after myelosuppressive therapy have had mixed results. After dose-intensive chemotherapy

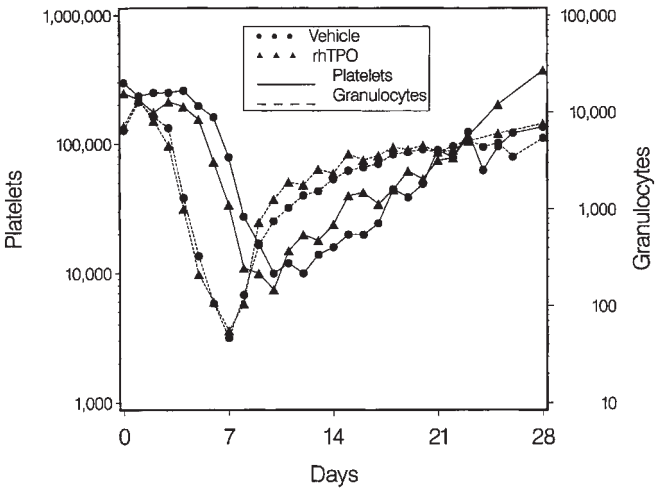


Figure 4. Effect of PEG-rhMGDF on platelet and granulocyte recovery after TBI (920 cGy) and allogeneic HSCT from DLA-identical littermates. No difference in time to recovery of platelets or granulocytes was observed between the 2 groups.

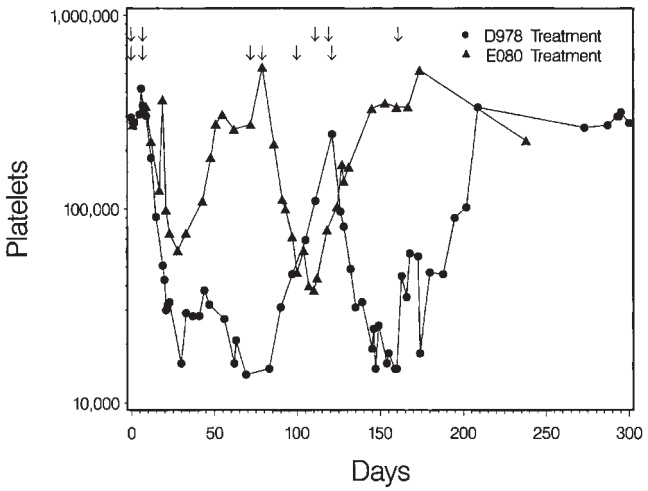


Figure 5. Retreatment of rhTPO-sensitized dogs to boost immune response. Two of 5 dogs were sensitized to rhTPO after TBI (200 cGy). The first retreatment with rhTPO of 10 μg/kg per day, 7 days apart for a total of 2 days, resulted in the recurrence of thrombocytopenia to a platelet count as low as 16,000/μL and 61,000/μL. The second retreatment with rhTPO of 5 μg/kg per day, 7 days apart for a total of 2 days of the 2 dogs (D978, E080) also resulted in the development of thrombocytopenia of a similar severity and duration. ↓ indicates treatment of sensitized dogs with rhTPO.

for solid tumors, the platelet nadir was increased and the duration of thrombocytopenia was reduced with c-mpl ligand therapy [35,36]. However, after induction chemotherapy for acute myelogenous leukemia, time to achieve a platelet count of at least 20,000/μL was not affected by c-mpl ligand therapy, although later platelet counts were increased in the treatment group [37,38]. The effectiveness of c-mpl ligand therapy after myelosuppression may depend on clinical factors, including type of disease, type and intensity of cytotoxic therapy, and the dose and schedule of recombinant c-mpl ligand.

Early studies in a murine HSCT model after otherwise lethal TBI showed that recombinant c-mpl ligand enhanced platelet recovery [39-41]. However, the administration of c-mpl ligand did not shorten the duration of severe thrombocytopenia or granulocytopenia in preclinical (large animals) or clinical studies of myeloablative therapy and autologous HSCT [42-46]. After allogeneic HSCT, additional risk factors for delayed platelet recovery include the presence of GVHD, HLA disparity, and therapeutic immunosuppression (eg, cyclosporine) [2,3]. Moreover, after allogeneic HSCT there is an increased risk of late failure of platelet recovery [1]. In spite of or because of these additional risk factors, no effect of c-mpl ligand therapy was observed on the time to platelet recovery after allogeneic HSCT in dogs. Although it would not be expected because c-mpl is not expressed on immunologically active cells, c-mpl ligand therapy was not associated with an increased risk of GVHD. No other preclinical studies of c-mpl ligand therapy after allogeneic HSCT have been reported in large animals or humans. A dose-finding clinical study in patients with significantly delayed platelet recovery has investigated rhTPO therapy after either autologous or allogeneic HSCT [47]. The start of rhTPO therapy was a median of 65 days (range, 40-129 days) after transplantation. Although this was a dose-finding study, a therapeutic effect of c-mpl ligand on platelet recovery was not readily apparent. No effect on the development of GVHD was noted.

In the PEG-rhMGDF study, episodes of liver necrosis occurred in the dogs after allogeneic stem cell transplantation. These episodes did not interfere with the evaluation of the effect of PEG-rhMGDF on platelet recovery because liver necrosis was diagnosed after platelet recovery in most cases. Events of liver necrosis occurred in both arms of the study, and therefore we concluded that it was not a toxicity of PEG-rhMGDF. The cause of the liver necrosis is uncertain. Cyclosporine is known to have toxic effects on the liver. However, this dose has been tolerated well in many previous studies of GVHD in this model. A viral cause was also considered because after this study was completed, confirmed episodes of viral hepatitis (herpes) were noted in the dog colony. The liver pathology was comparable to that observed with viral hepatitis.

The development of neutralizing antibodies against human TPO or human PEG-rhMGDF has been documented in 2 different preclinical studies. In the first study, chronic administration of PEG-rhMGDF to dogs resulted in a prolonged period of severe thrombocytopenia. Recovery of platelet counts to >100,000/μL from the nadir occurred over 3 months [23]. In a second study in mice, a TPO knock-out phenotype developed after neutralizing antibodies against human TPO were induced by the infusion of a recombinant adenovirus encoding human TPO [48]. A chronic immune thrombocytopenia persisted as long as 8 to 10 months after treatment. Treatment with TBI (500 cGy) before the infusion of the recombinant adenovirus encoding human TPO prevented the development of thrombocytopenia in some mice. In dogs, TBI (200 cGy) before rhTPO treatment was not sufficient to prevent sensitization and the development of thrombocytopenia. The development of sensitization to rhTPO in the dogs was likely promoted by the cross-species difference. It is possible that the risk of sensitization to rhTPO may be less with intravenous infusion than with subcutaneous injection. Plasma from the rhTPO-sensitized dog was infused into normal dogs; it resulted in a decreased platelet count, consistent with the conclusion that rhTPO-specific antibodies alone induce thrombocytopenia. Platelet survival studies were normal, indicating that the rhTPO-specific antibodies decreased the production of platelets or the release of platelets into the peripheral circulation.

The development of neutralizing antibodies with the resultant thrombocytopenia in preclinical models was predictive of complications in clinical trials. This observation is important because the likely mechanism for the development of thrombocytopenia is the production of antibodies to rhTPO that cross-reacted and neutralized the native canine form of TPO. Patients have developed neutralizing antibodies specific for PEG-rhMGDF in a study of chemotherapy-induced thrombocytopenia and in another study with normal individuals. Thrombocytopenia was noted to develop in some patients with the occurrence of the neutralizing antibodies [49-51]. The development of neutralizing antibodies and clinically significant thrombocytopenia in

some individuals after receiving PEG-rhMGDF resulted in the discontinuation of clinical trials of this agent in the United States [49]. To date, no rhTPO-specific neutralizing antibodies have been detected in any of the clinical trials (Beryl Hartley-Asp, Pharmacia, personal communication, February 2002).

In dogs, c-mpl ligand had a modest effect on recovery of platelet counts after low-dose myelosuppression but had no effect on platelet recovery after allogeneic HSCT. Sensitization to rhTPO after low-dose TBI resulted in transient but significant episodes of thrombocytopenia in some dogs. No neutralizing antibodies were detected after allogeneic marrow transplantation. The potential development of neutralizing antibodies and subsequent thrombocytopenia after low-dose myelosuppression may limit the clinical role of recombinant c-mpl ligands until further information is available on its prevention or until there is a high degree of certainty that it will not occur in humans with the type of c-mpl ligand being administered.

ACKNOWLEDGMENTS

This work was supported in part by grant DK42716 from the National Institute of Diabetes, Digestive and Kidney Diseases, grant CA15704 from the National Cancer Institute, and grant HL03701 from the National Heart, Lung and Blood Institute, National Institutes of Health, Department of Health and Human Services, Bethesda, MD; Amgen, Thousand Oaks, CA; and ZymoGenetics, Seattle, WA. Support was also received from the Josef Steiner Krebsstiftung, Bern, Switzerland. The ELISA to monitor for the presence of rhTPO-specific antibodies was performed by Amgen. The RIA and the neutralizing antibody bioassay for PEG-rhMGDF was performed by Zymogenetics.

The authors are grateful to the technicians of the Shared Canine Resource and the Hematology and Transplantation Biology Laboratories for their technical assistance. We thank Benjamin Weigler, DVM, PhD, and Michelle Spector, DVM, who are providing veterinary support for the dog colony. We are also grateful to Helen Crawford, Bonnie Larson, Lori Ausburn, Susan Carbonneau, and Karen Carbonneau for their excellent administrative support and Gary Schoch for the production of the figures for the manuscript.

REFERENCES

1. Bruno B, Gooley T, Sullivan KM, et al. Secondary failure of platelet recovery after hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2001;7:154-162.
2. Bernstein SH, Nademanee AP, Vose JM, et al. A multicenter study of platelet recovery and utilization in patients after myeloablative therapy and hematopoietic stem cell transplantation. *Blood*. 1998;91:3509-3517.
3. Nash RA, Gooley T, Davis C, Appelbaum FR. The problem of thrombocytopenia after hematopoietic stem cell transplantation [republished in *The Oncologist* 1996;1:371-380]. *Stem Cells*. 1996;14:261-273.
4. Gaydos LA, Freireich EJ, Mantel N. The quantitative relation between platelet count and hemorrhage in patients with acute leukemia. *N Engl J Med*. 1962;266:905-909.
5. Gmur J, Burger J, Schanz U, Fehr J, Schaffner A. Safety of stringent prophylactic platelet transfusion policy for patients with acute leukaemia. *Lancet*. 1991;338:1223-1226.
6. MacManus M, Lamborn K, Khan W, Varghese A, Graef L, Knox S. Radiotherapy-associated neutropenia and thrombocytopenia: analysis of risk factors and development of a predictive model [review]. *Blood*. 1997;89:2303-2310.
7. Hokom MM, Lacey D, Kinstler OB, et al. Pegylated megakaryocyte growth and development factor abrogates the lethal thrombocytopenia associated with carboplatin and irradiation in mice. *Blood*. 1995;86:4486-4492.
8. Neelis KJ, Visser TP, Dimjati W, et al. A single dose of thrombopoietin shortly after myelosuppressive total body irradiation prevents pancytopenia in mice by promoting short-term multilineage spleen-repopulating cells at the transient expense of bone marrow-repopulating cells. *Blood*. 1998;92:1586-1597.
9. Nash RA, Seidel K, Storb R, et al. Effects of rhIL-11 on normal dogs and after sublethal radiation. *Exp Hematol*. 1995;23:389-396.
10. Mouthon MA, Van der Meeren A, Gaugler MH, et al. Thrombopoietin promotes hematopoietic recovery and survival after high-dose whole body irradiation. *Int J Radiat Oncol Biol Phys*. 1999;43:867-875.
11. Kaushansky K. Thrombopoietin and the hematopoietic stem cell [review]. *Blood*. 1998;92:1-3.
12. Bunting S, Widmer R, Lipari T, et al. Normal platelets and megakaryocytes are produced in vivo in the absence of thrombopoietin. *Blood*. 1997;90:3423-3429.
13. Ballmaier M, Germeshausen M, Schulze H, et al. c-mpl mutations are the cause of congenital amegakaryocytic thrombocytopenia. *Blood*. 2001;97:139-146.
14. Arnold JT, Daw NC, Stenberg PE, Jayawardene D, Srivastava DK, Jackson CW. A single injection of pegylated murine megakaryocyte growth and development factor (MGDF) into mice is sufficient to produce a profound stimulation of megakaryocyte frequency, size, and ploidization. *Blood*. 1997;89:823-833.
15. Harker LA, Hunt P, Marzec UM, et al. Regulation of platelet production and function by megakaryocyte growth and development factor in nonhuman primates. *Blood*. 1996;87:1833-1844.
16. Ramsfjell V, Borge OJ, Veiby OP, et al. Thrombopoietin, but not erythropoietin, directly stimulates multilineage growth of primitive murine bone marrow progenitor cells in synergy with early acting cytokines: distinct interactions with the ligands for c-kit and FLT3. *Blood*. 1996;88:4481-4492.
17. Broudy VC, Lin NL, Fox N, Taga T, Saito M, Kaushansky K. Thrombopoietin stimulates colony-forming unit-megakaryocyte proliferation and megakaryocyte maturation independently of cytokines that signal through the gp130 receptor subunit. *Blood*. 1996;88:2026-2032.
18. Cramer EM, Norol F, Guichard J, et al. Ultrastructure of platelet formation by human megakaryocytes cultured with the Mpl ligand. *Blood*. 1997;89:2336-2346.
19. Akahori H, Shibuya K, Obuchi M, et al. Effect of recombinant human thrombopoietin in nonhuman primates with chemotherapy-induced thrombocytopenia. *Br J Haematol*. 1996;94:722-728.
20. Somlo G, Sniecinski I, ter Veer A, et al. Recombinant human thrombopoietin in combination with granulocyte colony-stimulating factor enhances mobilization of peripheral blood progenitor cells, increases peripheral blood platelet concentration, and accelerates hematopoietic recovery following high-dose chemotherapy. *Blood*. 1999;93:2798-2806.

21. Ulich TR, del Castillo J, Yin S, et al. Megakaryocyte growth and development factor ameliorates carboplatin-induced thrombocytopenia in mice. *Blood*. 1995;86:971-976.
22. Storb R, Raff RF, Graham T, et al. Marrow toxicity of fractionated versus single dose total body irradiation is identical in a canine model. *Int J Radiat Oncol Biol Phys*. 1993;26:275-283.
23. Dale DC, Nichol JL, Rich DA, et al. Chronic thrombocytopenia is induced in dogs by development of cross-reacting antibodies to the MpL ligand. *Blood*. 1997;90:3456-3461.
24. Burnett RC, Francisco LV, DeRose SA, Storb R, Ostrander EA. Identification and characterization of a highly polymorphic microsatellite marker within the canine MHC class I region. *Mamm Genome*. 1995;6:684-685.
25. Wagner JL, Burnett RC, DeRose SA, Francisco LV, Storb R, Ostrander EA. Histocompatibility testing of dog families with highly polymorphic microsatellite markers. *Transplantation*. 1996;62:876-877.
26. Wagner JL, Burnett RC, Works JD, Storb R. Molecular analysis of DLA-DRBB1 polymorphism. *Tissue Antigens*. 1996;48:554-561.
27. Nash RA, Burstein SA, Storb R, et al. Thrombocytopenia in dogs induced by granulocyte-macrophage colony-stimulating factor: increased destruction of circulating platelets. *Blood*. 1995;86:1765-1775.
28. Cox DR, Hinkley DV. *Theoretical Statistics*. London, England: Chapman & Hall; 1974.
29. Efron B, Tibshirani RJ. *An Introduction to the Bootstrap*. New York, NY: Chapman & Hall; 1993.
30. Siegel S, Castellan NJ Jr. *Nonparametric Statistics for the Behavioral Sciences*. New York, NY: McGraw-Hill; 1988.
31. Farese AM, Hunt P, Grab LB, MacVittie TJ. Combined administration of recombinant human megakaryocyte growth and development factor and granulocyte colony-stimulating factor enhances multilineage hematopoietic reconstitution in nonhuman primates after radiation-induced marrow aplasia. *J Clin Invest*. 1996;97:2145-2151.
32. Kaushansky K, Broudy VC, Grossmann A, et al. Thrombopoietin expands erythroid progenitors, increases red cell production, and enhances erythroid recovery after myelosuppressive therapy. *J Clin Invest*. 1995;96:1683-1687.
33. Neelis KJ, Qingliang L, Thomas GR, Cohen BL, Eaton DL, Wagemaker G. Prevention of thrombocytopenia by thrombopoietin in myelosuppressed rhesus monkeys accompanied by prominent erythropoietic stimulation and iron depletion. *Blood*. 1997;90:58-63.
34. Neelis KJ, Dubbelman YD, Qingliang L, Thomas GR, Eaton DL, Wagemaker G. Simultaneous administration of TPO and G-CSF after cytoreductive treatment of rhesus monkeys prevents thrombocytopenia, accelerates platelet and red cell reconstitution, alleviates neutropenia, and promotes the recovery of immature bone marrow cells. *Exp Hematol*. 1997;25:1084-1093.
35. Vadhan-Raj S, Verschraegen CF, Bueso-Ramos C, et al. Recombinant human thrombopoietin attenuates carboplatin-induced severe thrombocytopenia and the need for platelet transfusions in patients with gynecologic cancer. *Ann Intern Med*. 2000;132:364-368.
36. Basser RL, Underhill C, Davis I, et al. Enhancement of plateletrecovery after myelosuppressive chemotherapy by recombinant human megakaryocyte growth and development factor in patients with advanced cancer. *J Clin Oncol*. 2000;18:2852-2861.
37. Schiffer CA, Miller K, Larson RA, et al. A double-blind, placebo-controlled trial of pegylated recombinant human megakaryocyte growth and development factor as an adjunct to induction and consolidation therapy for patients with acute myeloid leukemia. *Blood*. 2000;95:2530-2535.
38. Archimbaud E, Ottmann OG, Yin JA, et al. A randomized, double-blind, placebo-controlled study with pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF) as an adjunct to chemotherapy for adults with de novo acute myeloid leukemia. *Blood*. 1999;94:3694-3701.
39. Molineux G, Hartley C, McElroy P, McCrea C, McNiece IK. Megakaryocyte growth and development factor accelerates platelet recovery in peripheral blood progenitor cell transplant recipients. *Blood*. 1996;88:366-376.
40. Molineux G, Hartley CA, McElroy P, McCrea C, McNiece IK. Megakaryocyte growth and development factor stimulates enhanced platelet recovery in mice after bone marrow transplantation. *Blood*. 1996;88:1509-1514.
41. Fibbe WE, Heemskerk DP, Laterveer L, et al. Accelerated reconstitution of platelets and erythrocytes after syngeneic transplantation of bone marrow cells derived from thrombopoietin pretreated donor mice. *Blood*. 1995;86:3308-3313.
42. Hartong SC, Neelis KJ, Visser TP, Wagemaker G. Lack of efficacy of thrombopoietin and granulocyte-macrophage colony-stimulating factor after total body irradiation and autologous bone marrow transplantation in Rhesus monkeys. *Exp Hematol*. 2000;28:753-759.
43. Wagemaker G, Neelis KJ, Hartong SC, et al. The efficacy of recombinant thrombopoietin in murine and nonhuman primate models for radiation-induced myelosuppression and stem cell transplantation [review]. *Stem Cells*. 1998;16:375-386.
44. Bolwell B, Vredenburgh J, Overmoyer B, et al. Phase 1 study of pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF) in breast cancer patients after autologous peripheral blood progenitor cell (PBPC) transplantation. *Bone Marrow Transplant*. 2000;26:141-145.
45. Wolff SN, Herzig R, Lynch J, et al. Recombinant human thrombopoietin (rhTPO) after autologous bone marrow transplantation: a phase I pharmacokinetic and pharmacodynamic study. *Bone Marrow Transplant*. 2001;27:261-268.
46. Fields KK, Crump M, Bence-Bruckler I, et al. Use of PEG-rHuMGDF in platelet engraftment after autologous stem cell transplantation. *Bone Marrow Transplant*. 2000;26:1083-1088.
47. Nash RA, Kurzrock R, DiPersio J, et al. A phase I trial of recombinant human thrombopoietin in patients with delayed platelet recovery after hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2000;6:25-34.
48. Abina MA, Tulliez M, Duffour MT, et al. Thrombopoietin (TPO) knockout phenotype induced by cross-reactive antibodies against TPO following injection of mice with recombinant adenovirus encoding human TPO. *J Immunol*. 1998;160:4481-4489.
49. Vadhan-Raj S. Clinical experience with recombinant human thrombopoietin in chemotherapy-induced thrombocytopenia [review]. *Semin Hematol*. 2000;37:28-34.
50. Yang C, Xia Y, Li J, Kuter DJ. The appearance of anti-thrombopoietin antibody and circulating thrombopoietin-IgG complexes in a patient developing thrombocytopenia after the injection of PEG-rHuMGDF [abstract]. *Blood.* 1999;94 (suppl 1): 681a. Abstract 3014.
51. Crawford J, Glaspy J, Belani C, et al. A randomized, placebo-controlled, blinded, dose scheduling trial of pegylated recombinant human megakaryocyte growth and development factor (PEG-RHUMGDF) with filgrastim support in non-small cell lung cancer (NSCLC) patients treated with paclitaxel and carboplatin during multiple cycles of chemotherapy [abstract]. *Program/Proceedings American Society of Clinical Oncology.* 1998; 17:73a. Abstract 285.